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Ubiquitin, a central component of selective cytoplasmic proteolysis, is linked to proteins residing at the locus of non-selective proteolysis, the vacuole

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Ubiquitin, an evolutionary highly conserved protein, is known to be involved in selective proteolysis in the cytoplasm. Here we show that ubiquitin–protein conjugates are also found in the yeast vacuole. Mutants defective in the major vacuolar endopeptidases, proteinase yscA and yscB, lead to accumulation of ubiquitin–protein conjugates in this cellular organelle.

Proteolysis; Ubiquitin; Vacuole; Lysosome; Yeast; *Saccharomyces cerevisiae*

1. INTRODUCTION

Ubiquitin, a polypeptide of 76 amino acids, has been found to play a major role in non-lysosomal proteolysis [1,2]. In an ATP-requiring process, ubiquitin is conjugated onto proteins to be degraded [1,2]. A major cytoplasmic proteinase, called proteasome or multicatalytic/multifunctional proteinase, is a component of this degradative process: mutants in the yeast proteasome (proteinase yscE) were found to accumulate proteins which are destined to be degraded via the ubiquitin pathway ([3], Richter-Ruoff, B., Heinemeyer, W. and Wolf, D.H., in preparation). Surprisingly, ubiquitin had been found in autophagic vacuoles and lysosomes of hepatoma cells [4]. In yeast, the equivalent of the lysosome is the vacuole [5–9]. The proteolytic properties of the vacuole of the yeast, *Saccharomyces cerevisiae*, are well characterized. Mutants of the major proteolytic activities of this organelle are available [5,7,8]. Experiments using mutants deficient in the two vacuolar endopeptidases, proteinase yscA and yscB, unraveled the central function of the vacuole in protein degradation under conditions of nutritional stress: 85% of total cellular degradation is due to the action of proteinase yscA and yscB [10]. The proteinase yscA- and yscB-deficient mutants may provide information about the proteins delivered to the vacuole as, due to defect of these two major proteolytic activities, these proteins might be prevented from degradation. We analyzed proteinase yscA and yscB mutant strains for ubiquitin–protein conjugates.

Abbreviations: IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; v, volume; w, weight; w/o, without.

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2. MATERIALS AND METHODS

2.1. *Yeast strains and media*

Strains used were: YS18 (*Matα his3-11,15 leu2-3,112 ura3Δ5 can*); YHH32 (*Matα his3-11,15 leu2-3,112 ura3Δ5 can pra1::URA3 prb1::ΔAV*) [11].

Growth of cells was performed either in mineral medium (MV, 0.67% yeast nitrogen base without amino acids, 2% glucose and auxotrophic nutrients, 30 mg/l each) followed by starvation for nitrogen in mineral medium without nitrogen (0.17% yeast nitrogen base w/o amino acids, w/o ammonium sulfate, 2% glucose, and auxotrophic nutrients, 30 mg/l each) or in complete liquid medium (YPD, 1% yeast extract, 2% peptone, 2% glucose).

2.2. *Starvation for nitrogen*

Cells were grown exponentially in mineral medium (2×10^7 cells/ml), collected by centrifugation, washed twice and resuspended in mineral medium without nitrogen. Aliquots of cells were harvested by centrifugation after 0 and 24 h of incubation in mineral medium without nitrogen.

2.3. *Isolation of vacuoles*

Isolation of vacuoles was performed as described by Wiemken et al. [12] with modifications according to Mechler et al. [13]. Cells were grown in complete medium until 3 h after loss of glucose. Spheroplast formation was performed using zymolyase and was followed by spheroplast lysis by DEAE-dextran. Vacuoles were isolated from spheroplast lysate by two subsequent sucrose gradient centrifugations.

Vacuolar preparations were characteristically enriched in the vacuolar marker enzyme α-mannosidase (27–30-fold for strain YS18, 9–12-fold for strain YHH32) (measured according to [14]). Vacuolar fractions showed negligible activity of the cytosolic marker enzyme glucose-6-phosphate dehydrogenase [15] and no activity at all of the mitochondrial marker enzyme succinate dehydrogenase [16]. The activity of an enzyme from secretory vesicles, acid phosphatase [17], was

slightly reduced in vacuolar fractions when compared to spheroplast lysate.

2.4. *Alkaline sodium carbonate fractionation*

Vacuolar fractions were diluted 50-fold in ice-cold 100 mM sodium carbonate solution, pH 11.5, as described by Roberts et al. [18]. After 30 min on ice the mixture was centrifuged at 100,000 × *g*. The supernatant was neutralized with 1 N acetic acid and precipitated in 5% trichloroacetic acid on ice. Precipitated protein was pelleted, washed twice with diethylether, dried, resuspended in sample buffer for electrophoresis and heated at 70°C until dissolved. The sediment was homogenized in sample buffer and also heated at 70°C until complete solubilization had occurred.

2.5. *Preparation of cell extracts, electrophoresis and immunoblot*

For preparation of cell extracts, cultures were rapidly chilled to 4°C, washed and resuspended in ice-cold 0.1 M potassium phosphate buffer to yield a 30% (w/v) solution. Cells were broken by vortexing with glassbeads (0.5 mm diameter, 1/3 of volume of cell suspension) for 5 × 1 min with intermittent chilling. Cell lysates were centrifuged for 5 min at 10,000 × *g* in the cold. Aliquots of the supernatants (40 μg of protein as measured according to Lowry [19]) were separated by SDS-polyacrylamide gel electrophoresis (10% gels, [20]) and blotted onto nitrocellulose [21]. Non-specific binding of antisera to nitrocellulose was prevented by incubation of filters (5 × 7 cm) in 0.05% Tween 20, 0.9% NaCl, 40 mM Tris–HCl, pH 7.4 (coating buffer) for at least 1 h, then treated overnight with a 1/500 dilution of anti-ubiquitin–protein conjugate in 10 ml of coating buffer. After washing twice in coating buffer peroxidase-coupled goat anti-rabbit IgG antibody was added and binding was detected by addition of 4-chloro-1-naphthol.

Visualization of total protein in electrophoresis gels was done by silver staining as described by Heukeshoven and Dernick [22].

2.6. *Antibodies against ubiquitin–protein conjugates*

Antisera used were either a generous gift of A. Ciechanover, Haifa, Israel, or raised by ourselves. Antibodies were generated by immunization of rabbits with a SDS-denatured ubiquitin–γ-globulin crosslink product and affinity-purified using ubiquitin–sepharose according to Hershko et al. [23]. Affinity-purified antibodies recognize ubiquitin–protein conjugates generated by incubation of Fraction II of reticulocyte lysate with ATP and ubiquitin on immunoblots. Antisera do not recognize Fraction II of reticulocyte lysate incubated with ubiquitin in the absence of ATP [24]. Antibody 33 also reacts to some extent with free ubiquitin. Antigenic reaction with yeast proteins was suppressed when pre-incubating antisera with ubiquitin–protein conjugates (50 μg) of Fraction II.

2.7. *Electron microscopy*

Cells were harvested by centrifugation, washed twice with distilled water and resuspended in a solution of 3% glutaraldehyde (EM grade) in 100 mM phosphate buffer, pH 7.2. Suspensions were kept on ice for 90 min. Excess glutaraldehyde was then removed by washing twice with phosphate buffer. For morphological studies intact cells were fixed in potassium permanganate [25]. For immunocytochemistry, after dehydration in a graded ethanol series cells were embedded in Lowicryl K4M. Immunocytochemical experiments were performed on ultrathin sections using the protein A/gold method [26].

3. RESULTS

Mutants deficient in proteinase yscA and proteinase yscB activity accumulate proteolytic substrates in their vacuole. Growing on mineral medium, the mutant cells (strain YHH32, *pra1::URA3, prb1::ΔAV*) exhibit a single, large vacuole filled with aggregated material and some vesicles (Fig. 1b). After starvation of the mutant cells in nitrogen-free medium, the number of vesicles dramatically increases (Fig. 1c). In contrast, wild-type cells growing on mineral medium exhibit a large vacuole that only contains aggregates (Fig. 1a). During starvation on nitrogen-free medium, the wild-type vacuole remains in this state (not shown). We analyzed crude extracts of mutant strain YHH32 (*pra1::URA3 prb1::ΔAV*), and, for control, of wild-type cells for ubiquitin–protein conjugates. In wild-type cells growing actively on mineral medium or incubated on nitrogen starvation medium ubiquitin–protein conjugates are hardly detectable in extracts (Fig. 2a,b). In contrast, in the proteinase yscA and yscB double mutant strain ubiquitin–protein conjugates increase considerably upon incubation on nitrogen starvation medium (Fig. 2c,d). This indicated that ubiquitin–protein conjugates might be part of the proteolytic substrates accumulating in the vacuole of the proteinase yscA and yscB mutant strains during starvation for nitrogen as shown in Fig. 1. In contrast to wild-type (Fig. 2e), accumulation of ubiquitin–protein conjugates also occurs in the proteinase yscA and yscB mutant strains grown in complete medium into stationary growth phase (Fig. 2f).

The yeast strains used in these experiments are isogenic except for the mutations in the genes of proteinase yscA (*pra1::URA3*) and proteinase yscB (*prb1::ΔAV*). Therefore the accumulation of ubiquitin–protein conjugates in the proteinase yscA and yscB double mutant strain (YHH32; Fig. 2d and f) must be due to the lack of activity of the two vacuolar endopeptidases, proteinase yscA and yscB. If ubiquitin–protein conjugates were to be degraded in the hydrolytic organelle of the vacuole (lysosome), these conjugates should accumulate in the vacuoles of mutants defective in vacuolar protein degradation.

Vacuoles were isolated from strains grown in complete medium as in Fig. 2e and f. Immunoblots of vacuolar protein of proteinase yscA and yscB double mutant cells show a bulk of high molecular weight proteins recognized by the affinity-purified antibody to ubiquitin–protein conjugates (Fig. 3b). Wild-type vacuolar protein only weakly reacts. In addition, the antigenic wild-type vacuolar proteins are of lower molecular weight when compared to the mutant proteins (Fig. 3a). For comparison of ubiquitin–protein conjugates of mutant and wild-type vacuoles the same amount of protein was applied on the gels, as can be seen after silver staining in a gel (Fig. 3c,d) run in parallel to that used for Western blot analysis (Fig. 3a,b).

Accumulation of ubiquitin–protein conjugates in the vacuoles of proteinase yscA- and yscB-deficient cells is also shown by immunocytochemistry (Fig. 4). In wild-type cells two affinity-purified antisera to ubiquitin–protein conjugates show staining of the nucleus and cytoplasm and some antigenic reaction of vacuolar contents (Fig. 4a,b). Proteinase yscA and yscB double mutant cells show ubiquitin–protein conjugates in the nu-

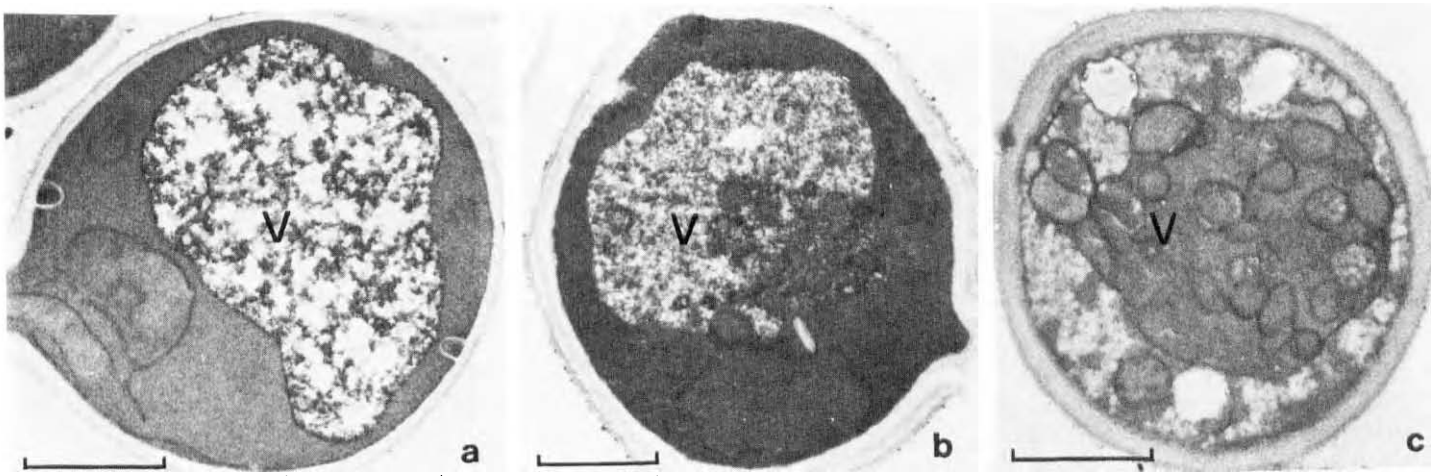

Fig. 1. Vacuole image of wild-type and proteinase mutant cells as viewed by electron microscopy. Cells were harvested and prepared for electron microscopy using potassium permanganate as described [25]. (a) Strain YS18 (wild-type) grown on mineral medium (cell density 2×10^7 cells/ml). (b) Strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB grown on mineral medium (cell density 2×10^7 cells/ml). (c) Strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB grown on mineral medium (cell density 2×10^7 cells/ml) followed by incubation on mineral medium without nitrogen (24 h). V, vacuole.

cleus, the cytoplasm and an especially large amount of antigenic material in the vacuole (Fig. 4c,d).

As ubiquitin–protein conjugates of the vacuole appear to be connected to vacuolar aggregates (Fig. 4) we checked whether they were of membraneous origin. For this purpose vacuoles of proteinases yscA and yscB double mutant cells were treated with 100 mM sodium carbonate, pH 11.5, and centrifuged at 100,000 × *g*. Under these conditions, intrinsic membrane proteins pellet with the membrane fraction, whereas soluble and peripheral membrane proteins are recovered in the supernatant [27,28]. As can be seen in Fig. 5, ubiquitin–protein conjugates of the vacuole are mainly insoluble after sodium carbonate treatment. This might indicate a membraneous origin of the conjugates. However, one should note that ubiquitin–protein conjugates might

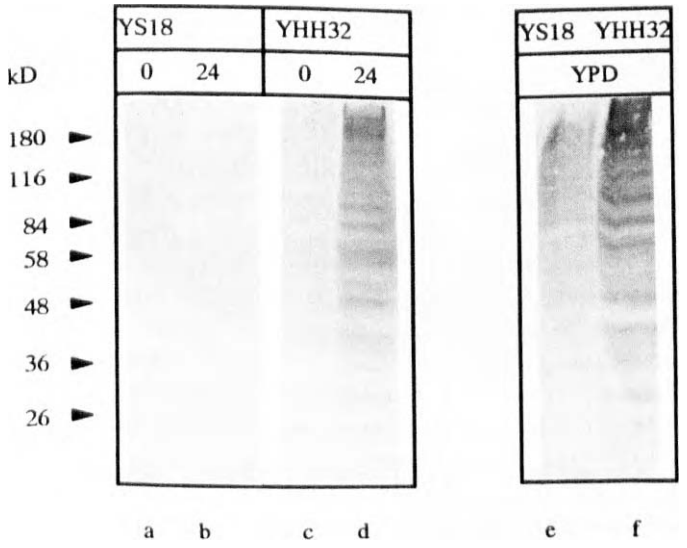

Fig. 2. Immunoblot of ubiquitin–protein conjugates in cell extracts during starvation. Cells grown on mineral medium (final cell density 2×10^7 cells/ml) were incubated on mineral medium without nitrogen for 0 and 24 h. Alternatively, cells were grown on complete medium into stationary phase. Cells were harvested and cell extracts were prepared as described in Materials and Methods. Extracts (40 μg of protein per lane) were subjected to electrophoresis and subsequent immunoblotting using antibody 15. (Lanes a and b) Extracts of wild-type strain YS18 after 0 and 24 h of incubation in mineral medium without nitrogen. (Lanes c and d) Extracts of strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB after 0 and 24 h of incubation in mineral medium without nitrogen. (Lanes e and f) Extracts of wild-type strain YS18 and strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB grown on complete medium until 3 h after loss of glucose.

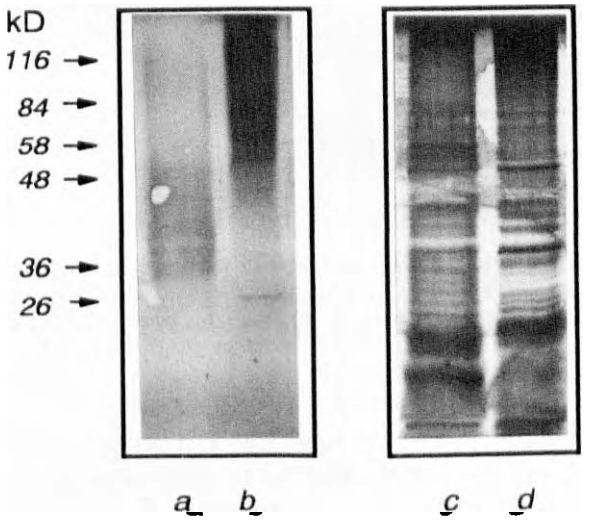

Fig. 3. Immunoblot of ubiquitin–protein conjugates in isolated vacuoles. Vacuoles were isolated from stationary phase cells according to [12,13]. Vacuolar protein (25 μg per lane) was subjected to electrophoresis and immunoblotting using antibody 15 (lanes a,b). Total protein content was visualized by silver stain according to [22] (lanes c,d). (Lanes a,c) Vacuoles of strain YS18 (wild-type). (Lanes b,d) Vacuoles of strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB.

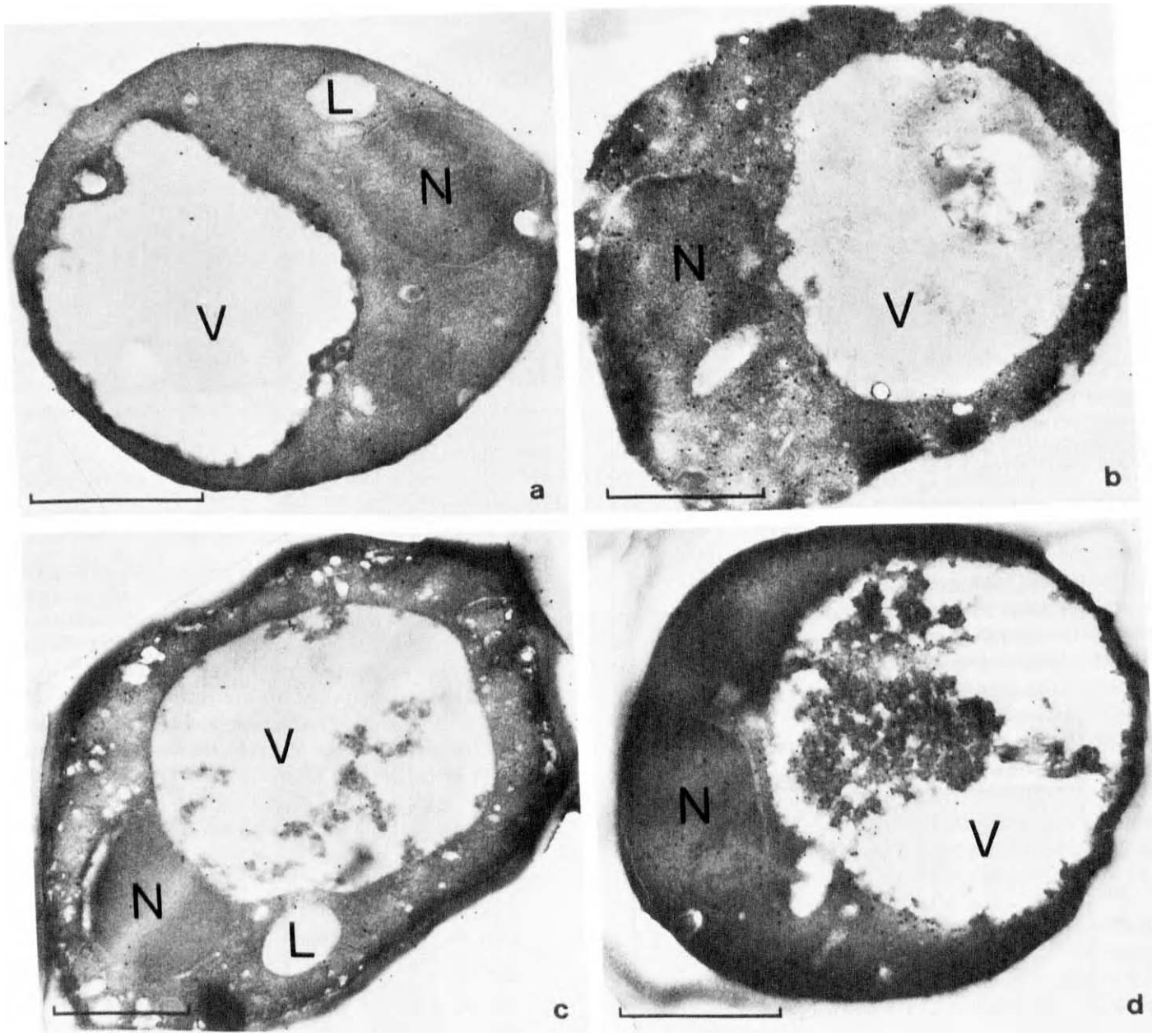

Fig. 4. Ubiquitin–protein conjugates in wild-type and proteinase mutant cells as viewed by immunogold electron microscopy. Cells grown on complete medium into stationary growth phase were harvested, fixed with glutaraldehyde and prepared for immunogold electron microscopy according to [25,26]. Lanes a,c, antibody 15; lanes b,d, antibody 33. Lanes a,b, strain YS18 (wild-type); lanes c,d, strain YHH32 (*pra1::URA3 prb1::ΔA1*) deficient in proteinases yscA and yscB. L, lipid droplet; N, nucleus; V, vacuole. Bar = 1 μm.

have a tendency to form aggregates under vacuolar conditions and may thus behave like membrane proteins.

4. DISCUSSION

Using yeast strains defective in the two major vacuolar peptidases, proteinase yscA and yscB, we have shown that ubiquitin–protein conjugates accumulate in the vacuole (Figs. 3 and 4). This represents direct evidence that ubiquitin–protein conjugates are proteolytic substrates of this organelle. The protein moiety of vacuolar ubiquitin–protein conjugates must be rapidly degraded, as our antibodies detect only some ubiquitin–protein conjugates of lower molecular weight in the vacuoles of wild-type cells (Fig. 3a and Fig. 4a,b). Recently Gropper et al. [29] presented evidence of activated ubiquitin involved in stress-induced vacuolar proteolysis of the mouse cell cycle mutant, ts85. The observation of Schwartz et al. [4], who detected free ubiquitin in the lysosome of hepatoma cells, might reflect the

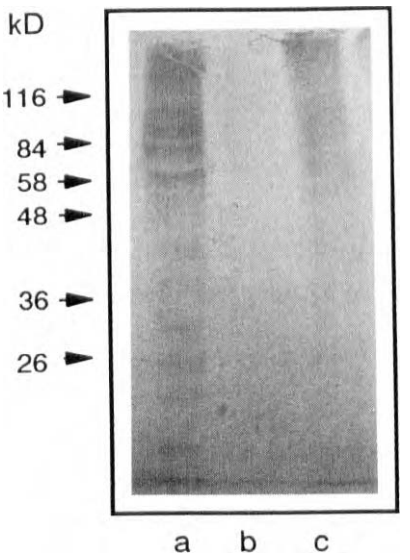

Fig. 5. Immunoblot of ubiquitin–protein conjugates after alkaline sodium carbonate fractionation of isolated vacuoles. Vacuolar protein (50 μg) of strain YHH32 (*pra1::URA3 prb1::ΔAV*) deficient in proteinases yscA and yscB was subjected to incubation in alkaline sodium carbonate and subsequent centrifugation as described [18]. (Lane a) Untreated vacuoles. (Lane b) Supernatant of fractionation. (Lane c) Sediment of fractionation.

stability of free ubiquitin towards proteolytic digestion in this organelle of which the yeast vacuole is an equivalent. At least the major portion of ubiquitin–protein conjugates accumulating in proteinase yscA and yscB mutant cells are insoluble after treatment with sodium carbonate (Fig. 5). This indicates that vacuolar ubiquitin–protein conjugates may be integral membrane proteins. Mayer and colleagues [30] report the accumulation of ubiquitin-protein conjugates in the lysosomal system of fibroblasts after long-term treatment with cysteine protease inhibitors. These ubiquitin–protein conjugates are insoluble in Triton X-100 and potassium iodide solution. Subcellular fractionation of reticulocyte extracts revealed that 25% of total ubiquitin–protein conjugates of these cells sedimented with the 22,000 × *g* stromal fraction [24], and ubiquitin was found at the cell surface conjugated to several receptor proteins [31–33]. As plasma membrane proteins are thought to be degraded by lysosomal mechanisms [34], ubiquitin may serve as a signal or (and) denaturing detergent for making the respective proteins susceptible for degradation in this organelle. However, our experiments cannot rule out that the sodium carbonate-insoluble ubiquinated material found in the vacuole, at least in part, represents aggregates of formerly soluble proteins of other cellular compartments.

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